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Short communication

Ribosomal RNA-based analysis of the bacterial flora from the conjunctivae of cattle with bovine keratoconjunctivitis (BKC)

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Abstract

Bovine keratoconjunctivitis (BKC), colloquially referred to as 'pinkeye', is a disease affecting cattle worldwide; it costs cattle producers millions of dollars in economic loss annually. While *Moraxella* spp. are the primary etiologic agent of pinkeye, surveys of flora from the conjunctivae of livestock from around the world have indicated that a variety of bacterial commensals occupy this niche. We used molecular biology-based methods to determine the composition of bacterial flora in the conjunctivae of normal dairy and beef cattle from Maryland ($n = 113$), and beef cattle with clinical BKC from Louisiana ($n = 42$). Three regimens were used: 16S rRNA PCR and DGGE analysis of amplicons; 16S rRNA PCR and cloning of amplicons into *Escherichia coli* followed by screening and sequencing of clones harboring inserts; and culture of bacteria on chromogenic agar followed by 16S rRNA PCR and sequencing. Most taxa were comprised of saprophytes found in the environment, such as *Bacillus*, *Pantoea*, *E. coli*, and *Exiguobacterium*. *Moraxella* spp. were infrequently observed. Some species, such as *Propionibacterium acnes*, represent taxa not previously associated with the conjunctivae. *Bacillus pumilus* and *Bacillus licheniformis* isolates from the conjunctivae of Maryland cattle were genetically distinct from isolates previously implicated in septic infections in cattle at the same location. We conclude that employing 16S rRNA-based methods for bacterial identification can be useful in defining the flora present in the conjunctivae of normal cattle, and those with BKC.
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Keywords: Bovine keratoconjunctivitis (BKC); Pinkeye; Conjunctivae; Bacterial flora

1. Introduction

Conjunctivitis, colloquially referred to as 'pinkeye', is an important cause of morbidity in cattle worldwide. Pinkeye can be caused by bacterial, viral, or nematode infection, trauma, and exposure to sunlight. A common clinical manifestation of pinkeye is bovine keratoconjunctivitis (BKC), caused by the

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Gram-negative, aerobic diplococcobacillus, *Moraxella bovis*. Outbreaks of BKC may achieve 80% morbidity and often compromise animal production for 2–6 weeks. It is estimated that in the United States alone, BKC affects more than 10 million head of cattle annually, resulting in a loss of 110–150 million dollars to the cattle industry (Henson and Grumbles, 1960; Nagy et al., 1989; Brown et al., 1998; Yeruham et al., 2001). Control of BKC can involve the use of commercial vaccine preparations derived from *M. bovis* (Smith et al., 1990; Conceicao et al., 2004), fly control, reduction of taller brush in pastures, and use of antibiotic ophthalmic ointment.

The means by which *M. bovis* gains access to the conjunctivae is not completely understood, but is thought to involve mechanical transmission by flies (Brown et al., 1998). The pathophysiology of BKC may be mediated in some degree by the presence of cytotoxic factors elaborated by *M. bovis* (Beard and Moore, 1994).

Previous studies evaluating normal and pathogenic ocular flora have been published for a variety of animal species from varied locales (Moore et al., 1988; Gionfriddo et al., 1991; Davidson et al., 1994; Cooper et al., 2001; Pinard et al., 2002; Rosa et al., 2003; Prado et al., 2005). Culturable organisms reported in these investigations include *Bacillus*, *Staphylococcus*, *Streptococcus*, *Moraxella*, and *Branhamella*. Whether bacterial commensals present in the conjunctivae play a role in mediating BKC and *M. bovis* infection is not well understood. Accordingly, we were interested in identifying bacterial commensals present in the conjunctivae of cattle in Maryland and in Louisiana, including cattle with BKC. In carrying out our researches, we utilized three protocols.

One technique used involves amplification of DNA extracted from conjunctival swabs using conserved 16S rRNA primers, followed by separation of amplicons using denaturing gradient gel electrophoresis (DGGE), and the sequencing of bands excised from the gel. Another approach involves pooling BKC, and normal animal, swab DNAs, amplifying with conserved 16S rRNA primers, and cloning these sets of amplicons into *Escherichia coli* to create 'conjunctival flora libraries' which can then be subjected to sequence analysis. A third approach is to culture bacteria recovered from swabs using a variety of different chromogenic agar substrates; colonies with different colors and morphologies could then be selected for identification via 16S rRNA sequencing.

2. Materials and methods

2.1. Animals

Cattle sampled in Maryland were beef cattle and dairy cattle maintained on the campus of the Beltsville Agricultural Research Center (BARC) in Beltsville, MD. The beef cattle were of Angus/Hereford foundation, and housed on open pasture. The dairy cattle were registered Holsteins with confined, comfort-stall housing. Cattle ranged in age from 3 to 48 months and were sampled at periodic intervals from late Fall 2005 to December 2006. Most animals received an annual BKC vaccination using a commercially available vaccine (Pinkeye Shield XT4, Novartis Animal Health, Inc., Larchwood, IA, USA), however, cattle sampled in October and December 2006 did not receive vaccinations. During the study period there were no cases of clinical BKC in any of the cattle on the Beltsville campus, and thus none of the sampled animals had received any treatment for pinkeye (i.e., ophthalmic antibiotic ointments).

Cattle sampled in Louisiana were a subset of a herd of 350 Holstein steers, between 8 and 10 months of age and weighing ~500 lb (227 kg), purchased from several sources in Mississippi, commingled, and shipped to Louisiana in April, 2005. Upon arrival, the steers were rotationally grazed on Bermuda grass pastures of 80–160 acres with free-choice water and minerals available in each pasture. The steers had fence-line contact in some of the pastures with a 150 head crossbred cow-calf herd with Brahman influence. None of these Brahman cattle exhibited clinical signs of BKC. Other herds of crossbred cattle were also pastured within 100 yards of the steers. BKC incidence in these herds was unknown.

A large number (~150) of the steers displayed clinical signs of BKC in June 2005, including excessive lacrimation, photophobia, corneal edema or corneal ulceration. Conjunctival swabs were obtained at this time; steers with corneal ulceration were designated as having BKC and $n = 42$ were

sampled. Six animals without corneal ulceration or other clinical signs of BKC were selected as normal controls. Subsequently, the entire herd received a dose of commercial BKC vaccine (Ocu-guard MB 1, Boehringer Ingelheim, Vetmedica, St. Joseph, MO, USA), fly tags (Patriot eartags, KMG Chemicals Co., Houston, TX, USA) were inserted, and an injection of vitamins A, D, & E (Veterinary Laboratories, Inc., Lenexa, KS, USA) was administered. Steers were also dewormed with a pour-on antiparasitic (Merial USA, Duluth, GA, USA). Additionally, BKC affected steers received an antibiotic injection administered subcutaneously (Oxytetracycline, 13.6 mg per lb body weight, 10 ml per injection site; Merial Ltd., Duluth, GA, USA) and a subconjunctival antibiotic injection (Penicillin G, 6×10^5 IU injected under the bulbar conjunctiva twice weekly; Phoenix Scientific, Inc., Ft. Dodge, IA, USA).

2.2. Sampling and DNA extraction

All conjunctival sampling of Maryland and Louisiana cattle was performed by veterinarians. Non-sterile latex gloves were worn during the sampling procedures. In Maryland cattle, sterile cotton swabs were used to sample the conjunctiva in one eye per randomly selected animal. Cattle were restrained in a squeeze chute, haltered, and their head made secure to one side. Eyelids were separated with the thumb and forefingers of one hand while the sterile cotton swab (Puritan Medical Products Co., Guilford, ME, USA) was placed in the ventral subconjunctival cul-de-sac, swirled two to three times and then placed promptly into sterile 15-ml conical centrifuge tubes containing 1 ml of Lysis Buffer (20 mM Tris–Cl pH 8.0, 2 mM EDTA, 1.2% Triton X-100 and 20 mg/ml lysozyme), or 1 ml of sterile H_2O, and hand delivered to the lab within 1–2 h of sampling.

For Louisiana cattle, the CultureSwab™ collection & transport system (BBL/Biotrace Intl., Bothell, WA, USA) was used. The eyelids were retracted and the swab was placed in the ventral subconjunctival cul-de-sac. The swab was replaced in the transport tube and placed on cold packs prior to express delivery to Beltsville. Immediately upon receipt at Beltsville, swabs destined for PCR (DGGE or cloning) were incubated, with periodic vortexing, at 70 °C for 10 min. The swab was then discarded and the tube's liquid contents were subjected to genomic DNA extraction using the DNAeasy Tissue kit (Qiagen, La Jolla, CA, USA) according to the manufacturer's recommendations. DNA was eluted in a volume of 50 μl and stored at 4 °C.

2.3. PCR and DGGE

PCR and DGGE analyses were conducted on swabs from 26 dairy cattle and 37 beef cattle, sampled in Maryland during the spring and summer of 2005, as well as 42 swabs from Louisiana cattle with BKC, and 6 swabs from normal Louisiana cattle, collected in June 2005. The 357f (GC-clamped; equivalent to nucleotides 341–357 of *E. coli*) and 518r (nucleotides 518–543 of *E. coli*) set of Yu and Morrison (2004), which amplify a ~190-bp segment of the 16S rRNA gene, and the 341f (GC-clamped; equivalent to nucleotides 341–357 of *E. coli*) and 907r (nucleotides 907–926) primer set of Casamayor et al. (2000), which amplify a ~585–625-bp segment of the 16S rRNA gene, were used. PCR reactions (50 μl volume) used 50 pmol each primer, 1.5 U *Taq* polymerase (Q-Biogene, Irvine, CA, USA) and 10 μl DNA extracted from swabs. Thermal cycling conditions were 2 min at 94 °C, then 30 cycles of 45 s at 94 °C; 45 s at 55 °C; 1 min at 72 °C. Successful amplification of PCR products was confirmed by electrophoresing a 10-μl aliquot on a 1% agarose gel with ethidium bromide staining. The remaining products were dried down in a vacuum centrifuge (Savant Instruments, Holbrook, NY, USA) and resuspended in 30 μl sterile water and stored at 4 °C until used for DGGE, which was performed on a dCode apparatus (Bio-Rad, Hercules, CA, USA). Gels were 12% acrylamide (8% acrylamide for the stacking gel) with a 60–40% denaturating gradient.

Electrophoresis was conducted at 200 V for ~6 h, after which gels were stained with a 1:10,000 dilution of SYBR Gold (Molecular Probes, Eugene, OR, USA). Bands of interest were cut from the gel, placed into 150 μl sterile water, and incubated overnight at 4 °C. A 5-μl aliquot was used as template for re-amplification with the 341 non-GC-clamped forward and 907r primer set. PCR products were electrophoresed on 4% agarose E-gels (Invitrogen, Gaithersburg, MD, USA) to confirm that reamplified products were a single band; if so, reamplified product was treated with

Exo-Sap (USB Scientific, Cleveland, OH, USA) (1.5 μl per 4 μl PCR product) and subjected to dye-terminator cycle sequencing reactions using the Big Dye™ 3.1 reagent (Applied Biosystems, Foster City, CA, USA) (5 pmol primer, 1 U Big Dye™ 3.1 master mix, 2 μl 5× Reaction Buffer, brought up to a 10-μl total volume with water). Sequencing products were then purified using isopropanol precipitation, and analyzed on an ABI 3100 model automated fluorescence sequencing instrument (Applied Biosystems, Foster City, CA, USA). If the re-amplified product displayed two bands on the E-gel, the more abundant one was excised from the gel and subjected to sequencing.

2.4. Cloning and library screening

In an alternate approach to characterizing the microbial flora of the conjunctivae from Maryland animals, 10-μl aliquots of DNA, selected at random from $n = 20$ of the panel of swab DNAs obtained from Maryland Holstein cattle sampled in spring and summer 2005, were pooled. For the samples from Louisiana cattle, 10-μl aliquots of DNA extracted from conjunctival swabs were pooled from $n = 8$ randomly selected BKC samples, as were aliquots of DNA obtained from all the non-BKC eye swabs ($n = 6$). The BKC and non-BKC pools were subjected to drying in a vacuum microcentrifuge and the dried nucleic acids reconstituted in 30 μl of sterile water. Twelve microlitres of each DNA pool was then subjected to amplification with the 341f (non-GC-clamped) and 907r primer set of Casamayor et al. (2000). The resultant band of PCR product was excised from a 2% agarose gel and the product extracted from the agarose using the QIAquick kit (Qiagen, La Jolla, CA, USA) and eluted into 50 μl of elution buffer. A 10 μl volume was re-examined on an agarose gel to confirm it was of sufficient size and intensity, after which the eluted DNA was ligated into pCR®-XL-TOPO plasmid and electroporated into TOP 10 (Invitrogen, Gaithersburg, MD, USA) electrocompetent *E. coli* cells via electroporation using a Gene Pulser II apparatus (Bio-Rad, Hercules, CA, USA) with 1.8 kV and 100 Ω. Transformed *E. coli* were selected on LB plates with kanamycin (50 μg/ml). Colonies were subcloned in Terrific Broth (Invitrogen, Gaithersburg, MD, USA) with kanamycin in 2 ml deep-well plates overnight at 37 °C. The cultures were then transferred to 96-well plates and plasmid DNA (pDNA) was extracted using either the RapidPURE® plasmid mini 96 kit (Q-Biogene, Irvine, CA, USA) or the PerfectPrep® kit (Eppendorf, Hamburg, Germany). pDNA was eluted in 50–100 μl elution buffer and stored at 4 °C until used.

Five to ten microlitres of pDNA was used as a template for PCR screening of insert-positive clones with M13 forward and reverse primers, with thermal cycling conditions of 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 s, 55 °C for 90 s, and 72 °C for 2 min. PCR products were electrophoresed on E-96™ gels (Invitrogen, Gaithersburg, MD, USA) and amplicons of the predicted size (∼600 bp) were considered to be indicative of insert-positive clones. PCR products were treated with Exo-Sap, with 2 μl treated product used as a template for sequencing reactions. Sequencing reactions were performed according to the manufacturer's instructions, sequencing products were then purified using isopropanol precipitation, and analyzed on an ABI 3100 model automated fluorescence sequencing instrument.

2.5. Culture of bacteria from swabs and 16S rRNA amplification

Another approach to characterizing the flora of the conjunctivae relied on culture of bacteria, followed by identification of the cultured organisms using 16S rRNA amplification and sequencing. All cattle assayed with this protocol were from Maryland. Swabs were obtained from Angus heifers and cows in March ($n = 12$) and April ($n = 10$) 2006 and Holstein heifers in October ($n = 12$) 2006, as well as unvaccinated Holstein heifers in December 2006 ($n = 16$).

Swabs were delivered to the laboratory within 1–2 h of sampling and 100-μl aliquots of the swab eluate were added to a 5 ml vial of chromogenic Coliscan® Easygel® nutrient agar (Micrology Laboratories, Goshen, IN, USA). After gentle mixing, the Easygel nutrient was poured onto Easygel plates and allowed to set at room temperature for ∼45 min. In an effort to use alternate means for selecting colonies of interest, 50–100 μl aliquots of the swab eluate was also plated on CHROMagar™ *E. coli* O157:H7 media (DRG International, Mountainside, NJ), and potato dextrose

agar (Accumedia Manufacturers, Lansing, MI, USA). The plates were then incubated overnight at 37 °C. The following morning the plates were examined for the presence of colonies of different colors and morphologies; colonies were sampled using a sterile inoculators' loop and DNA extracted from the bacterial cells using Instagene matrix (Bio-Rad, Hercules, CA, USA). For all colonies, except those cultured from the December 2006 conjunctival swab samples, amplification of most of the 16S rRNA gene (~1250–1400 bp) was performed using the primers of Kazor et al. (2003). The October and December 2006 swab sample-derived colonies were amplified using the 341f–907r primer set of Casamayor et al. (2000); this was performed because the analyses of sequences derived from the larger amplicons indicated that accurate assignation of isolates to genus level was feasible with shorter amplicons. Confirmation of amplification was done using 2% agarose gels, after which products were treated with Exo-Sap reagent and sequenced.

2.6. Sequence analysis

Only sequence files of good quality (i.e., distinct electropherogram peaks with minimal shoulder overlap with adjoining peaks, and minimal background) were analyzed using Sequencher™ (Gene Codes Corp., Ann Arbor, MI, USA) and submitted to the NCBI BLAST website for determination of the closest match from sequences residing in the bacterial 16S rRNA database. Sequencing, and designation of highest-scoring homologies among Genbank entries, was done throughout 2006. Taxa assigned to 16S rRNA sequences amplified from conjunctival swab DNAs, and bacterial isolates, represent those with the highest-scoring similarities based on BLAST alignments; in cases when there were a number of genera and/or species all with equivalent similarity scores, we chose the species with (presumably) greater likelihood of residing in the dairy farm, or animal tissue, environment.

2.7. Randomly amplified polymorphic DNA-PCR analysis of Bacillus isolates

The Giraffa and Rosetti protocol (2004) was performed for amplification of bacterial DNA from cultures of *Bacillus* spp. Briefly, 5 μl of Instagene-extracted bacterial DNA, obtained from chromogenic agar plate cultures, was subjected to PCR using the M13 minisatelite-sequence primer at 100 pmol, with 3.0 mM $MgCl_2$, 2 U DNA polymerase, and 10 mM each dNTP in a 50 μl reaction volume. Thermal cycling conditions were the same as those of Giraffa and Rosetti, except the extension step was increased to 2 min. Aliquots of PCR product were quantitated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies LLC, Wilmington, DE, USA) and ~400–500 ng (approximately 17 μl) were electrophoresed on 2% agarose gels.

2.8. Quality control of sequence data

To prevent the inclusion of chimeric sequences in our database, sequences that failed to display ≥94% homology with known Genbank depositions were removed from analysis. Sequences displaying >95% homology with Genbank depositions were examined for concatamerization using the program Chimera Check (Ribosomal Databse II website, url: rdp.cme.msu.edu/index.jsp); those displaying evidence of a chimeric nature were removed from analysis.

3. Results

3.1. DGGE analysis of conjunctiva flora

The quantities of DNA recovered from the Maryland dairy ($n = 26$) and beef ($n = 37$) cattle conjunctivae swabs, sampled in spring and summer 2005, was unmeasurable even with a NanoDrop™ microliter-scale spectrophotometer. However, use of conserved 16S rRNA primers did result in the amplification from these DNAs of bands of PCR products, which were then separated using DGGE. Sequence analyses of DGGE bands excised from gels receiving amplicons derived from 16S rRNA PCR products generated from Maryland beef heifers was disappointing. Most bands were faint and tended to yield sequences that were chimeras, or had poor sequence reads; there were seven sequences with the 357f/518r set (~190-bp product), that were of reasonable quality. Three of these were identified as

Table 1
Identities of DGGE bands derived from 16S rRNA PCR products generated from Louisiana Holstein steer conjunctival swabs obtained in June 2005

Source[a]	nt aligned[b]	BLAST identity	% Similarity	Accession No.
Normal	533	*Exiguobacterium* spp.	99	DQ019167
Normal	560	*Acinetobacter lwoffii*	99	DQ328322
Normal	559	*Bacillus silvestris*	99	AJ006086
Normal	559	*Stenotrophomonas maltophilia*	99	AJ011332
BKC	560	*Streptococcus plutanimalium*	99	Y18026
BKC	562	*Pseudomonas* sp. BRS-2	100	AB267069
BKC	560	*Moraxella osloensis*	99	AY730714
BKC	499	*Moraxella bovis*	99	AF005183
BKC	561	*Macrococcus equipercicus*	99	AM237362
BKC	559	*Pantoea agglom./Edwardsiella/Enterobacter*	99	
BKC	572	*Enterobacter cowanii/Edwardsiella tarda*	99	
BKC	510	*Pseudomonas* spp.	98	AY331352
BKC	561	*Exiguobacterium acetylicum*	99	DQ019167
BKC	478	*Pseudomonas umsongensis*	96	AY972277
BKC	559	*Planococcus* sp. Smarlab	99	AY538695
BKC	539	*Arthrobacter gandavensis*	99	AM237357
BKC	553	*Arthrobacter luteolus*	99	DQ486130

[a] BKC = bovine keratoconjunctivitis.
[b] Refers to length of sequence read derived from band excised from DGGE gel. Amplicons were generated using the 341f and 907r primer set of Casamayor et al. (2000).

Moraxella osloensis (93–100% homology with Genbank entry AF005190), one as *Clostridium bifermentans* (99%, Y18787), and one as *Streptomyces tumescens* (84%, AF346483). One sequence had low homology to that of a putative agent of rhinosporidiosis (61%, AJ440719), and another sequence had 77% homology to an uncultured bacterium from corn (X86563). Because some of the sampled animals had manure present in their conjunctivae when sampled (due to wet and rainy conditions during the sampling session), the possibility exists that some of the taxa we identified are of manure origin, and represent adventitious contaminants in the conjunctivae.

While conjunctival swab DNAs from Maryland dairy herd animals yielded 16S rRNA amplicons, and bands on DGGE gels, all sequences from these bands were of poor quality and/or chimeric origin.

Conjunctival bacterial biomass was greater in swabs obtained from Louisiana Holstein steers presenting with clinical BKC, as well as Louisiana steers not displaying any symptoms, than from the Maryland cattle. Greater intensity of bands and greater variety of bands on subsequent DGGE gels were observed. BLAST-derived bacterial identities for sequences from 4 bands representing normal flora and 13 bands representing BKC animals are summarized in Table 1. In addition to finding *M. bovis* and *M. osloensis*, a variety of saprophytes and skin commensals also were observed. Similarity to existing Genbank entries ranged from 96 to 100%.

Over the 6-month course of conducting our 16S rRNA PCR and DGGE assays, we observed on several occasions bands in the extraction control or the no-template controls. These bands were extracted and sequenced, providing reads of 560–619 nt aligned length. The extraction control band had 98% similarity to X91612, an uncultured bacterium. One assay in the no-template control band gave a similarity of 99% with an uncultured *Pseudomonas* sp. (AY270168), and three others had 98% similarity with an uncultured bacterium (X91612).

3.2. Cloning and sequencing of 16S rRNA PCR products derived from conjunctiva swabs

Thirty-five cloned inserts from the library constructed from swab contents generated from Maryland Holstein cattle ($n = 20$) sampled in the spring of 2005, were subjected to amplification and sequencing with

M13 primers. The insert sizes of these clones ranged from 520 to 740 nt aligned length. The majority of the inserts displayed relatively poor similarity to existing Genbank entries, and those showing similarity values of <96% were shown to be of chimeric origin by Chimera Check software; those entries with scores ≥96%, and not of chimeric origin, included *Propionibacterium acnes* (99%, AE017283) and *Enhydrobacter aerosaccus* (98%, AJ550856), a member of the Moraxellaceae.

Libraries were generated from pools of DNA from both non-clinical ($n = 6$) and clinical BKC ($n = 8$) Louisiana cattle swabs. A total of 55 cloned inserts from these libraries (32 from non-clinical BKC and 23 from clinical BKC), provided good sequence reads and were not chimeric. In addition, only three of these had Genbank similarity scores of <97%. Some of the clones appeared to contain inserts with similarity to 16S rRNA sequences deposited from libraries representing uncultured bacteria. Culturable genera were frequently encountered and included *Acinetobacter* and *Exiguobacterium*. Interestingly, *Aeromonas* was unique to the clinical BKC library (Fig. 1). *Moraxella* sequences were not observed in either normal or clinical BKC libraries using this technique.

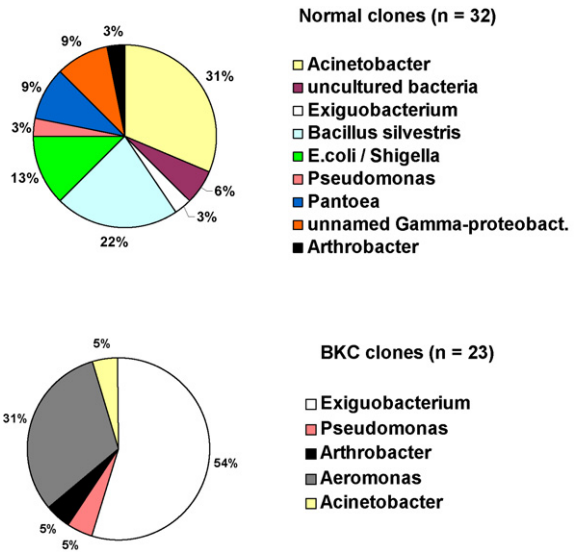

Fig. 1. Pie charts depicting proportions of bacterial taxa represented in libraries containing 16S rRNA PCR products amplified from conjunctival swabs taken from Louisiana Holstein steers with bovine keratoconjunctivitis (BKC), and normal steers (i.e., without BKC), in June 2005. 32 normal and 23 BKC clones were sequenced, with sequence lengths ranging from 619 to 740 nt in length. BLAST similarities ranged from 95 to 100%, with the majority of clones (49/55) providing ≥99% similarity.

3.3. Identity of bacteria cultured from conjunctival swabs

The paucity of bacterial DNA present in conjunctival swabs from Maryland cattle influenced us to attempt to culture bacterial flora from swabs, and then identify the resultant colonies via 16S rRNA sequencing. Culture plates receiving eluate from control swabs (i.e., swabs exposed for ∼15 s to the air of the sampling area, but not making contact with the animal) as well as agar-only controls, did not show any colonies after 48 h of incubation. After 24 h of incubation, most plates receiving conjunctiva swab eluate had colonies.

Nineteen colonies generated from 14 different Angus cattle, sampled in April 2006, were subjected to 16S rRNA PCR and sequencing of the resultant ∼1250–1400-bp amplicon. Twelve colonies originated from Chromagar plates (i.e., chromogenic plates designed for *E. coli* O157:H7 detection), and the remainder from Coliscan plates. Colony identifications are shown in Table 2. The species detected are all classifiable either as saprophytes or skin commensals, and all sequences had ≥97% similarity with existing Genbank entries. The majority of colonies growing on Coliscan plates were small (i.e., <2 mm diameter), red in color, and were identified as *Bacillus licheniformis* or *Bacillus pumilus*. Only colonies with blue coloration were found growing on the Chromagar plates. These colonies were identified as *Pantoea* and *Citrobacter*, as well as other members of the coliform group (Table 2).

When conjunctival swabs obtained from young unvaccinated Maryland Holsteins in October 2006 were cultured, some swabs did not generate bacterial growth on any of the plates. When colonies were present, they tended to be visibly fewer in number and of less morphologic variety when compared to colonies generated earlier in the year from swabs obtained from young vaccinated Angus. Of the 11 Holsteins whose swabs did generate bacterial growth, 19 colonies were subjected to 16S rRNA amplification and sequencing. No *Bacillus* was recovered and the majority of colonies (10 of 19, or 52%) had similarity

Table 2
Identities of bacteria cultured from the conjunctivae of Angus and Holstein cattle maintained at the Beltsville Agricultural Research Center in 2006

Origin of animal (H = Holstein, A = Angus)[a]	Highest-scoring BLAST identity	Length of aligned 16S rRNA nucleotide sequence[b]	% similarity with BLAST identity	Accession no.
A	*Staph. croceolyticus*	1319	100	AJ458194
A	*S. sciuri*	1071	98	AB212276
A	Enterobacteriaceae sp.	1300	98	AY538694
A	*Erwinia cypropredii*	1292	97	U80201
A	*Bacillus licheniformis*	1280	100	AF426469
A	*B. pumilus*	1206 ($n = 5$)	100	AY315440
A	*Citrobacter* spp.	1308	98	AF025370
A	*Klebsiella* spp.	1242	99	AY894126
A	*Enterococcus flavescens*	1389	100	Y12923
A	*Citrobacter farmeri*	1106	97	AF025371
A	*Pantoea* spp. A202	1197 ($n = 2$)	99	AY580079
A	*P. agglomerans*	606	97	AJ010097
A	*Enterobacter cloacae*	447	99	AY787819
H	*P. agglomerans*	615	99	AY924376
H	*A. lwoffi*	594	98	ALU10875
H	*Acinetobacter* spp.	269	99	DQ223676
H	*Pseudomonas* spp.	519 ($n = 2$)	98	AY850169
H	*Staphylococcus* spp.	366	95	EF061904
H	*S. lugdunensis*	413	99	DQ923426
H	*Pantoea* spp.	388 ($n = 2$)	94, 98	EF050809
H	*E. coli*/*Shigella* spp.	515 ($n = 10$)	97–99	DQ683069 AF527827 CP000468 EF032687

[a] Angus cattle were sampled in March and April 2006, Holsteins in October and December 2006. No animals displayed clinical signs of BKC.
[b] For multiple isolates, mean nucleotide lengths are provided, and the number of isolates sequenced. The primers of Kazor et al. (2003) were used to amplify and sequence a portion of the 16S rRNA gene for the isolates from Angus cattle, while the primers of Casamayor et al. (2000) were used for the isolates from the Holstein cattle.

to *E. coli* and *Shigella*. Other observed genera included *Pantoea*, *Staphylococcus*, and *Acinetobacter* (Table 2).

Effort was made to determine if the *B. licheniformis* and *B. pumilus* isolates obtained from Angus conjunctival swabs in March and April 2006 were genetically similar. RAPD-PCR was conducted on them, as well as on various *Bacillus* isolates previously acquired in February 2006 from tissues of a cow (No. 507) which died of septicemia at the Beltsville campus. Neither the *B. licheniformis* nor *B. pumilus* isolates from the conjunctivae displayed banding profiles similar to previously obtained isolates (Fig. 2, Panels A and B). Of the five *B. pumilus* conjunctivae isolates, those from animals 1128 and 1280 showed the closest degree of similarity, while the profiles of the other three isolates were more dissimilar (Fig. 2, Panel B). These results suggest that the *Bacillus* isolates recovered from the conjunctivae represent distinct lineages, and are unlikely to be adventitious contaminants of our sampling and DNA processing protocols.

Interestingly, <5 colonies were cultured from conjunctival swabs obtained from unvaccinated Maryland Holstein heifers in December of 2006, and these failed to provide amplicons of the proper size when assayed with a universal 16S rRNA primer set. When the remaining swab washings were plated onto potato dextrose agar culture plates, we observed the growth of large white and green colonies containing hyphae-like structures, suggestive of fungal organisms. These putative fungi were not present in control plates incubated in tandem with those receiving swab washings. DNA extracted from eight of these colonies was assayed using the universal fungal 18S rRNA primers of Paulino et al. (2006), as well as primers

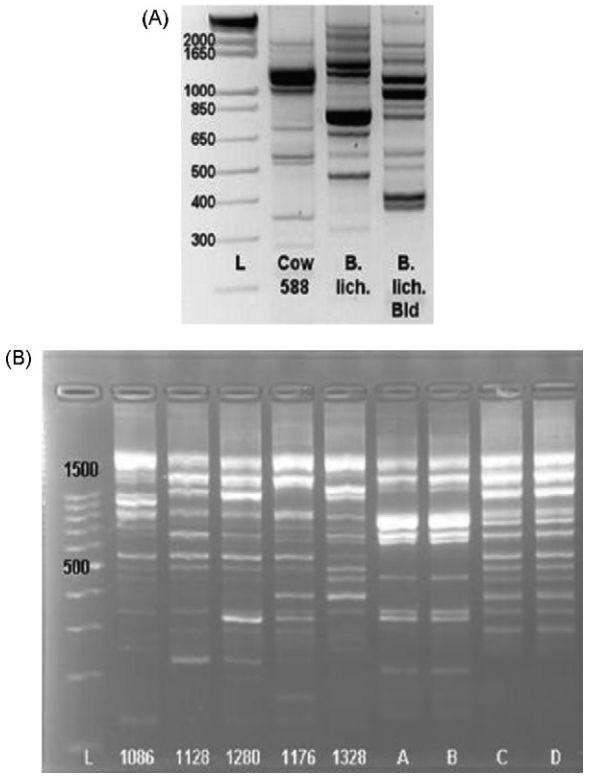

Fig. 2. Randomly amplified polymorphic DNA (RAPD)-PCR analysis of *Bacillus* spp. cultured from bovine conjunctivae, and *Bacillus* spp. cultured from tissues from a cow (No. 507) dying from septicemia at the Beltsville, Maryland, campus in February 2006. Panel A, results for *Bacillus licheniformis*. Lane L: DNA ladder with rung sizes indicated. Lane 'cow No. 588', *B. licheniformis* conjunctival isolate. Lane '*B. lich*': *B. licheniformis* milk isolate, cow No. 507. Lane '*B. lich* Bld': *B. licheniformis* blood isolate, cow No. 507. Panel B: results for *Bacillus pumilus*. Lane L: DNA ladder with rung sizes indicated. Lanes numbered 1086–1328: *B. pumilus* isolates from conjunctivae sampled in March and April 2006. Lanes A–D: *B. pumilus* isolates from tissues of cow No. 507.

designed for the amplification of a segment of the gene coding for the 65 kDa protein of Actinomycetes (Telenti et al., 1993). Two colonies, white and green, gave satisfactory amplicons with the fungal primers. Analysis of ~300 nt aligned length from these amplicons yielded 98% similarity with *Candida* (Genbank accession no. AY24225; white colony), and 98% similarity with *Filobasidium uniguttulatum* (Genbank accession no. AB032664; green colony). One colony, with a cream-like color and plaque-like morphology, gave an amplicon of predicted size with the Actinomycete primers. This colony was identified as *Nocardia farcinica* based on restriction enzyme digest patterns (Telenti et al., 1993). *N. farcinica* is the etiologic agent of bovine farcy (Steingrube et al., 1997).

4. Discussion

This study focused on two categories of animals: (1) a group consisting of young Holstein steers residing in Louisiana, sampled in summer 2005 when clinical signs of BKC were prevalent, but prior to vaccination for BKC and antibiotic therapy. (2) Groups of Angus and Angus/Hereford beef cattle, as well as Holstein dairy heifers and cows, residing in Maryland. With the exception of some of the Holsteins, all of these cattle had received one or more doses of BKC vaccination, as well as vaccinations for respiratory viruses and clostridia toxins. The cattle from Maryland were sampled at various times in 2005 and 2006, and BKC was never observed in any of these animals.

Conjunctival swabs from the Louisiana cohort were subjected to DGGE, and 16S rRNA library construction and sequencing. The documented BKC pathogen *Moraxella* was detected only by using the DGGE protocol. Many of the other taxa detected in BKC and non-BKC swabs using the 16S rRNA library technique were representative of saprophytes commonly encountered in the environment (Fig. 1).

While DGGE and 16S rRNA library approaches to categorizing the flora of Maryland cattle were less successful, we did observe the presence of some taxa, such as a *P. acnes* 16S rRNA amplicon (in a library constructed from swabs taken from BARC Angus cattle), that demonstrate the utility of a molecular-based approach to identification. This Gram-positive species is considered a slow-growing anaerobe and, since it requires specialized culture conditions, would not have been recovered in a conventional screening protocol. It has been associated, via 16S rRNA analysis, from the ocular surface of human patients suffering from 'dry eye' (Graham et al., 2007), but its presence in livestock has not been well-documented.

Since our efforts at using DGGE and 16S rRNA methods on conjunctival swabs obtained from Maryland Angus and Holstein cattle failed to generate the quantity of data we observed in the Louisiana cohort, we utilized culture, in conjunction with 16S rRNA sequencing, to identify bacteria present in the conjunctivae. We analyzed colonies derived from conjunctival swabs obtained from Maryland Holstein and Angus cattle in the spring, fall, and early winter of 2006. The majority of colonies sampled provided sequence identities of $\geq$97% similarity with existing Genbank accessions (Table 2).

The culture of *B. pumilus* and *B. licheniformis* from Angus cattle was particularly interesting because previous work had detected these organisms in tissues from a dairy cow on the Beltsville campus that died in February 2006 from a septic bacterial infection. *B. pumilus*, in particular, has been shown in previous reports to be a member of the udder flora of otherwise healthy dairy cows (Al-Qumber and Tagg, 2006). In an effort to determine if the *B. licheniformis* and *B. pumilus* cultured from the conjunctiva swabs were 'contaminants' from previous laboratory and clinical cultures, a RAPD-PCR genotyping was performed (Fig. 2). Results indicated that the conjunctival *Bacillus* isolates do not represent lineages already present on the dairy and/or in the research laboratory environment, and suggest these lineages may be unique to the conjunctival flora in sampled animals.

Many of the bacterial taxa identified in the conjunctiva samples in this study are among those previously reported to reside in the conjunctivae of companion animals and livestock. In their study on the flora of healthy calves, Barber et al. (1986) cultured *M. bovis*, *Branhamella catarrhalis* and *Mycoplasma bovoculi*. In their survey of stabled and hospitalized horses, Moore et al. (1988) recovered *Bacillus*, *Staphylococcus*, *Streptomyces*, *Neisseria*, *Moraxella*, and *Acinetobacter*. In normal pigs, Davidson et al. (1994) reported *Streptococcus* and *Staphylococcus* as the most prevalent genera. Gionfriddo et al. (1991) recovered *Staphylococcus* and *Pseudomonas* spp. from the conjunctivae of llamas, while Prado et al. (2005) reported the presence of *E. coli*, *Klebsiella*, *Pseudomonas*, and *Acinetobacter* in the eyes of dogs examined in veterinary clinics in northeastern Brazil.

Our study indicates that molecular methods may have some utility for determination of bacterial flora associated with BKC, as well as providing new information on the diversity of prokaryotes in the normal conjunctivae; as best as we can determine, this is the first report in the literature of the use of such methods for the latter purpose. The success of these methods is obviously dependent on the amount and quality of bacterial DNA that can be recovered from the conjunctivae of sampled animals. In our hands, 16S rRNA amplification and DGGE analyses were most successful when conducted on animals with clinical BKC and (presumably) a larger amount of bacterial biomass resident in the conjunctivae. In the event that culture is used to characterize the bacterial flora, 16S rRNA sequencing can be of use in assigning species identification with a degree of accuracy that can exceed that of traditional culture and biochemical-based protocols (Clarridge, 2004). The protocols reported here may prove useful to investigators pursuing the role of infectious agents in ophthalmic diseases in livestock.

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